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DOI:

[10.1111/bph.13638](https://doi.org/10.1111/bph.13638)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Powell, AD, Grafton, G, Roberts, A, Larkin, S, O'Neill, N, Palandri, J, Otvos, R, Cooper, AJ, Ulens, C & Barnes, NM 2016, 'Novel mechanism of modulation at a ligand-gated ion channel; action of 5-Cl-indole at the 5-HT_{3A} Receptor', *British Journal of Pharmacology*, vol. 173, no. 24, pp. 3467-3479. <https://doi.org/10.1111/bph.13638>

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Checked 8/11/2016

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Novel mechanism of modulation at a ligand-gated ion channel; action of 5-Cl-indole at the 5-HT₃A Receptor

Cryptic Orthosteric Modulation of 5-HT₃A

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Keywords

5-hydroxytryptamine-3 receptor /5-chloro-indole /ago-allosteric modulator /serotonin /pentameric ligand-gated ion channel / cryptic orthosteric modulator (COM)

Abstract

Background and purpose

The 5-HT₃ receptor is a prototypical member of the Cys-loop ligand-gated ion channel (LGIC) superfamily and an established therapeutic target. In addition to activation via the orthosteric site, receptor function can be modulated by allosteric ligands. We have investigated the pharmacological action of Cl-indole upon the 5-HT_{3A} receptor and identified that this positive allosteric modulator possesses a novel mechanism of action for LGICs.

Experimental approach

The impact of Cl-indole upon the 5-HT₃ receptor was assessed using single cell electrophysiological recordings and [³H]granisetron binding with HEK293 cells stably expressing the 5-HT₃ receptor.

Key results

Cl-indole failed to evoke 5-HT_{3A} receptor mediated responses (up to 30 µM) or display affinity for the [³H]granisetron binding site. However, in the presence of Cl-indole, termination of 5-HT application revealed tail currents mediated via the 5-HT_{3A} receptor that were independent of the preceding 5-HT concentration but were antagonised by the 5-HT₃ receptor antagonist, ondansetron. These tail currents were absent in the 5-HT_{3AB} receptor. Furthermore, the presence of 5-HT revealed a concentration-dependent increase in the affinity of Cl-indole for the orthosteric binding site of the h5-HT_{3A} receptor.

Conclusions and implications

Cl-indole acts as both an orthosteric agonist and an allosteric modulator but the presence of an orthosteric agonist (e.g. 5-HT) is a prerequisite to reveal both actions. Precedent for ago-allosteric action is available yet the essential additional presence of an orthosteric agonist is now reported for the first time. This widening of the pharmacological mechanisms to modulate LGICs may offer further therapeutic opportunities.

Tables of Links

Targets
5-HT₃ Receptors^a
5-HT₃A
5-HT₃AB

Ligands
[³H]-granisetron: IUPAC name 1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]nonan-7-yl)indazole-3-carboxamide
5-hydroxytryptamine: IUPAC name 3-(2-Aminoethyl)-1H-indol-5-ol
5-methylindole: IUPAC name 5-methyl-1H-indole. Not found in ligand database
5-hydroxyindole: IUPAC name 1H-Indol-5-ol
5-chloroindole: IUPAC name 5-chloro-1H-indole. Not found in ligand database
Ondansetron: IUPAC name 9-methyl-3-[(2-methylimidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4-one
Phenylbiguanide: IUPAC name 1-(diaminomethylidene)-2-phenylguanidine

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to Pharmacology (Southan et al., 2016), and are permanently archived in The Concise Guide to Pharmacology 2015/16 (^aAlexander et al., 2015)

Abbreviations

Cl-indole: 5-chloroindole; LGIC: ligand-gated ion channel; nAChR: nicotinic acetylcholine receptor; COM: cryptic orthosteric modulator; 5-HT: 5-hydroxytryptamine; 5OHI: 5-hydroxyindole; 5MeI: 5-methoxyindole

Introduction

The 5-HT₃ receptor is an excitatory ligand-gated ion channel (LGIC) of the Cys-loop family that includes the nicotinic acetylcholine receptors (nAChR), glycine and GABA_A receptors, and Zn²⁺-activated receptors (Barnes *et al.*, 2009; daCosta *et al.*, 2013). Members of this LGIC family share a common pentameric structure, an observation recently confirmed by the publication of the X-ray structure of the mouse 5-HT_{3A} receptor (Hassaine *et al.*, 2014). Each subunit of the 5-HT₃ receptor is composed of an extracellular N-terminal domain, four transmembrane domains and a short extracellular C-terminal domain. The orthosteric binding site is located at the interface between two adjacent N-terminal domains (daCosta *et al.*, 2013).

Five human 5-HT₃ receptor subunits have been identified (5-HT_{3A} to 5-HT_{3E}) (Karnovsky *et al.*, 2003; Niesler *et al.*, 2003). The native receptor can exist as a simple homopentamer of 5-HT_{3A} subunits or heteropentamers containing the 5-HT_{3A} subunit and at least one other of the 5-HT_{3B} to E subunits (Niesler, 2011). The pentameric structure gives rise to a potential for five ligand binding sites but recent evidence suggests that just two or three sites are occupied for maximal activation of the channel (Corradi *et al.*, 2009). Studies using heteromeric 5-HT_{3AB} receptors suggest that orthosteric binding sites are only formed between adjacent 5-HT_{3A} subunits (Lochner *et al.*, 2010; Thompson *et al.*, 2011).

As well as orthosteric agonism, allosteric modulation of the 5-HT₃ receptor has been reported by compounds as diverse as alcohols (Bentley *et al.*, 1998; Downie *et al.*, 1995; Stevens *et al.*, 2005), cannabinoids (Barann *et al.*, 2002), indole derivatives (Newman *et al.*, 2013; Parker *et al.*, 1996; Van Hooft *et al.*, 1997), terpenes (Lansdell *et al.*, 2015; Ziemba *et al.*, 2015), benzamides (Jørgensen *et al.*, 2011) and the class of novel negative allosteric modulators typified by PU02 (Trattinig *et al.*, 2012). A number of these modulators appear to have their effect via a site (or sites) in the transmembrane domain (e.g. for the indole derivatives (Hu *et al.*, 2008)). Allosteric agonism by phenolic monoterpenes has also been reported recently again via an allosteric transmembrane site (Lansdell *et al.*, 2015). An additional potential level of

allosteric modulation has been demonstrated since mutation of the intracellular portal region of the receptor affected gating and co-operativity (e.g. (Kozuska *et al.*, 2014)). This raises the possibility of receptor interactions with intracellular proteins leading to allosteric modulation of receptor function.

Whilst the majority of these allosteric effects are mediated by discrete, non-orthosteric sites, it is becoming apparent that some indole derivatives have a more intriguing and mixed mode of action. In particular, they may have mixed effects depending on their concentration and structure. For instance, the non-selective positive allosteric modulator, 5-hydroxy-indole (5OHI) displays positive allosteric modulation via a transmembrane domain of mouse 5-HT₃A receptors (Hu *et al.*, 2008), and can also display orthosteric agonism and allosteric inverse agonism at a spontaneously active mutated mouse 5-HT₃A receptor. These differing actions were dependent only on concentration. The closely related molecule, 5-methoxy-indole (5MeI) displayed both allosteric agonism and orthosteric inverse agonism at the same receptor, again dependent on concentration (Hu, 2015). Importantly the presence of the orthosteric ligand was not required for these actions.

We have recently described a selective allosteric modulator of the 5-HT₃ receptor, 5-chloro-indole (Cl-indole; (Newman *et al.*, 2013). Our study suggested that Cl-indole slows the decay of 5-HT₃A receptor currents and that it can reactivate desensitised 5-HT₃A receptors in the continued presence of 5-HT (Newman *et al.*, 2013). In the light of the mixed modes of action described for indole derivatives, we undertook a study to further explore the mode of action of Cl-indole on the modulation of the 5-HT₃ receptor. We identified for the first time within the LGIC superfamily, an ago-
allosteric modulation in which both agonism and allosteric modulation require the presence of the orthosteric ligand.

Materials and Methods

Nomenclature

The molecular target nomenclature conforms to the British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander *et al.*, 2015).

Cell Culture

HEK293 cells stably expressing the human 5-HT_{3A} subunit (HEK293-5-HT_{3A} cells (Brady *et al.*, 2001)) or the heteromeric 5-HT_{3A}/5-HT_{3B} receptor (HEK293-5-HT_{3A}/3B cells) (Dubin *et al.*, 1999) were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin (10,000 U ml⁻¹ penicillin and 10 mg ml⁻¹ streptomycin) and G418 (250 µg ml⁻¹) or G418 plus zeocin (250 µg ml⁻¹ each, HEK293-5-HT_{3A}/3B cells) and maintained at 37°C, 5% CO₂, 95% air at 95% relative humidity.

Radioligand binding

Radioligand binding assays were performed similar to our previous studies (*e.g.* (Monk *et al.*, 2004)). Briefly, cells were homogenised (Polytron) and resuspended in Tris buffer (in mM; Tris 25, pH 7.4 adjusted with NaOH). Radioligand binding assays were performed in triplicate; binding tubes contained 500 µl of competing drug(s) or vehicle (Tris buffer), and 100 µl of [³H]-granisetron (~1 nM); ~3 TBqmmol⁻¹; Perkin-Elmer). An aliquot (100 µl) of the cell homogenate was added to initiate binding, which was allowed to proceed at room temperature for 60 min before termination by rapid filtration and washing with Tris buffer under vacuum through Whatman GF/B filters, followed by assay of the radioactivity remaining on the filters.

5HT-Binding Protein

The A1B2D1_w 5HT-binding protein was produced and binding experiments with the protein were performed exactly as described by (Kesters *et al.*, 2013). Radioactivity was determined using a Top Count Scintillation and Luminescence reader (Packard).

Single cell electrophysiology

Approximately 18h prior to electrophysiology assays, cells were seeded directly onto 13 mm diameter glass coverslips coated with poly-L-lysine and fibronectin, at a density of 2×10^4 cells per coverslip. Macroscopic currents were recorded in the whole-cell recording mode of the patch-clamp technique from HEK293-5-HT3A cells or HEK293-5-HT3A/3B cells cultured on coverslips using an inverted microscope. Cells were superfused at $\sim 4 \text{ ml min}^{-1}$ with an extracellular solution (in mM; NaCl 140, KCl 2.8, CaCl₂ 1.0, glucose 10, HEPES 10, pH 7.4 adjusted with NaOH). Patch electrodes were pulled from borosilicate glass (O.D. 1.2 mm, I.D. 0.69 mm; Harvard Apparatus, Edenbridge, UK) using a P-97 puller (Sutter, Novato, CA) and filled with intracellular solution consisting of (in mM) 135 CsCl, 2 MgCl₂, 10 HEPES, 1 EGTA, 2 Mg-ATP and 0.3 Na-GTP; pH adjusted to 7.3 with KOH (osmolarity $\sim 285 \text{ mOsm}$). Patch electrodes typically had open tip resistances of 4-7 M Ω . Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Wokingham, UK), low-pass Bessel filtered at 1 kHz (NL-125, Digitimer Ltd, Welwyn Garden City, UK) and digitized at 10 kHz by a digidata 1302 (Molecular Devices). Experiments were performed at room temperature with the cells voltage-clamped at -60 mV.

Agonist-evoked currents were elicited by pressure ejection (20-kPa; Picospritzer II; General Valve, Fairfield, NJ) of agonist (5-HT 1.0-10 μM) from modified patch pipettes placed $\sim 30 \mu\text{m}$ from the recorded cell.

Data and Statistical Analysis

Values are expressed as mean \pm SEM. Curve fitting and data analysis were performed in Origin 8 (Silverdale Scientific, Stoke Mandeville, UK). Experimenters were not blinded to the experimental conditions. Experiments were randomised where appropriate (eg for the paired pulse experiments, interpulse interval was

randomised using a latin square design). ANOVA with subsequent unpaired t-tests (with Bonferonni correction for multiple comparisons) were used to evaluate differences; a Shapiro-Wilk tested normality. Post-hoc tests were only conducted if F was <0.05 ; the variance of the data was homogenous. Significance criterion was $p<0.05$.

Data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

CI-Indole modulates 5-HT-induced responses but is not an agonist

A brief application of CI-indole (30 μ M, 100 ms) did not evoke a current in HEK293 cells expressing the human 5-HT_{3A} receptor (Figure 1) whereas bath application of 5-HT (10 μ M) produced a large inward current. As the 5-HT-induced current began to decay, a second brief application of CI-indole (30 μ M, 100 ms) reactivated the current (Figure 1). These data demonstrate the CI-indole is not an agonist in its own right, but in the presence of 5-HT, it is capable of modulation of the 5-HT_{3A} receptor. The reactivation of the current suggested that CI-indole might act via an effect on either desensitisation or resensitisation of the receptor.

CI-Indole does not modulate 5-HT_{3A} receptor desensitisation

In order to establish if CI-indole modulates receptor desensitisation, we tested the effect of CI-indole on the rate of receptor desensitisation evoked by prolonged receptor activation. Twenty second applications of 5-HT (10 μ M) produced large currents (1.53 ± 0.25 nA, $n=11$) that displayed profound desensitisation ($12.4 \pm 3.23\%$ of peak; Figure 2Ai). The current decay was best fit with a double exponential function, with time constants of 1.02 ± 0.09 s and 9.79 ± 1.09 s (Figure 2Bi & Bii). Superfusion of CI-indole (10 μ M) did not alter the amplitude of 5-HT induced currents (1.37 ± 0.27 nA, $n=11$, $p=0.54$); nor did it alter the degree ($13.9 \pm 3.9\%$ of peak, $p=0.79$) or rate (τ_1 1.22 ± 0.20 s, $p=0.31$; τ_2 7.78 ± 0.41 s, $p=0.21$) of receptor desensitisation (Figure 2Biii). These data demonstrate that CI-indole does not modulate 5-HT_{3A} receptor desensitisation.

CI-indole does not modulate 5-HT_{3A} receptor resensitisation

We previously demonstrated that when 5-HT_{3A} receptors were completely desensitised by prolonged application of 5-HT, subsequent application of CI-indole generated an inward current indicative of CI-indole being able to reactivate desensitised receptors (Newman et al., 2013) (see also Figure 1). We therefore

tested whether CI-indole could modulate receptor resensitisation using a dual pulse protocol; a single prolonged application of 5-HT (10 μ M, 20 s) induced receptor desensitisation (Figure 3A). After removal of the 5-HT, a second test pulse of 5-HT (10 μ M, 1 s) was applied with a delay between 5 s and 2 minutes. Recovery from desensitisation was estimated by the fractional recovery of the current elicited by the second 5-HT pulse and was plotted against the inter-pulse interval. CI-indole did not modulate the rate of receptor resensitisation (control $\tau_r=18.4\pm2.0$ s; CI-indole $\tau_r=17.7\pm3.1$ s; n=5, p=0.8; Figure 3B).

CI-indole induces tail currents

In the absence of CI-indole, removal of 5-HT resulted in the evoked currents decaying back to the resting holding current level (see Figure 3A and 4A, *black traces*). In contrast, removal of 5-HT in the continued presence of CI-indole resulted in a relatively large, slowly developing inward tail current which peaked at 158.3 ± 30.1 pA ($13.3\pm1.8\%$) of the 5-HT-induced current amplitude (Figure 4A, red trace; n=13; see also Figure 3A, red trace). The rise time of the tail current was best fit by a single time constant ($\tau=2.77\pm0.35$ s) and the tail current decayed slowly back to baseline (time to reach 50% level 11.06 ± 1.7 s). The effect of CI-indole on the presence of a tail current was completely reversed upon wash (Figure 4A, *grey trace*). As the tail current arose immediately after the removal of the 5-HT, we examined if the tail current amplitude was correlated with the current remaining at the end of the agonist application; we also used lower concentrations of 5-HT (1 - 3 μ M and 1 μ M) as these lower 5-HT concentrations evoked currents with reduced desensitisation (Figure 4B). The tail current amplitude did not correlate with the amount of current remaining (Figure 4Bi, n=27, ANOVA p=0.32). In contrast, the amplitude of the tail current correlated positively with the 5-HT₃A receptor current density (Figure 4Bii, n=13, ANOVA p<0.001).

In order to further explore the origin of the tail current, we examined the current-voltage relationship of the tail current. Similar to the IV curve for the 5-HT₃A receptor current, the IV curve for the tail current reversed at approximately zero mV (Figure 5). These data suggest that the tail current arises from activation of 5-HT₃A

channels (see also figure 7), and that channel opens in a manner consistent with orthosteric receptor activation.

The tail current recorded in the presence of Cl-indole was qualitatively similar to tail currents that arise following relief from open channel block (Rossokhin et al., 2014). Given that 5-HT is known to induce channel block at concentrations above 5 μ M (Corradi et al., 2009), we explored further if Cl-indole could induce tail currents after application of lower concentrations of 5-HT. Cl-indole did not potentiate 3 μ M 5-HT-induced currents (Control = 0.73 ± 0.11 nA; Cl-indole = 0.68 ± 0.17 nA; $n=6$, $p=0.91$), yet the presence of Cl-indole induced a tail current upon removal of the lower concentration of 5-HT (Figure 6). Following removal of 5-HT at 3 μ M, the tail current peaked at 135 ± 29.8 pA (18.7 ± 3.3 % of the agonist induced current; $n=6$, $p=0.15$), with a similar decay time (8.54 ± 1.1 s; $p=0.65$). The rise time in the tail current (5.42 ± 0.78 s) following removal of 5-HT at 3 μ M was significantly slower than for tail currents evoked following removal of 10 μ M 5-HT ($p=0.01$, Figure 6).

Cl-indole (10 μ M) potentiated 1.0 μ M 5-HT-induced currents (Control = 149.0 ± 50.7 pA; Cl-indole = 473.5 ± 146.0 pA; $n=9$, $p=0.03$) but not currents arising from higher concentrations of 5-HT. In the presence of Cl-indole (10 μ M), tail currents were also observed following removal of 5-HT at 1 μ M; the peak amplitude of the tail current was 197.5 ± 50.6 pA, the rise time was significantly slower (7.37 ± 0.81 s; $p<0.001$) and the decay time was unaltered (13.73 ± 1.0 s; $n=9$, $p=0.26$; Figure 6). Although the peak amplitude of the tail current evoked by removal of 1 μ M 5-HT did not significantly differ from those evident following removal of higher 5-HT concentrations, the Cl-indole-induced tail currents evoked following removal of 1 μ M 5-HT were significantly larger *relative* to the 5-HT-induced peak current (108.2 ± 37.3 %; $n=9$, $p=0.003$).

Does Cl-indole function as an ago-allosteric modulator?

The homomeric 5-HT₃A receptor has 5 potential orthosteric binding sites, but various studies have shown that even when fully activated only 3 molecules of 5-HT are bound per receptor leaving unbound orthosteric sites (Corradi et al., 2009; Mott et al., 2001; Solt et al., 2007). Given the structural similarity between Cl-indole and the

natural ligand 5-HT, we hypothesised that Cl-indole may function as an ago-allosteric modulator, a mode of action which has previously been described in members of the GPCR superfamily (Schwartz *et al.*, 2006). We tested this in two ways: firstly, we examined if the competitive antagonist, ondansetron, which interacts with the orthosteric site, could block the Cl-indole-induced tail current. Secondly, we examined if 5-HT could modulate the ability of Cl-indole to interact with the orthosteric site in radioligand binding studies.

In the continual presence of Cl-indole, ondansetron (10 μ M) was applied via a second picospritz pipette one second prior to the withdrawal of the 5-HT. In the absence of ondansetron, tail current amplitude was 250 ± 18 pA, whereas effectively no tail current was observed in the presence of ondansetron (11 ± 3 pA; $n = 5$, $p < 0.001$ Figure 7A). In a parallel set of experiments, we tested the effect of ondansetron (10 μ M) applied after development of the tail current. Ondansetron applied at the peak of the tail current immediately reduced the current back to baseline levels (Figure 7B, $n = 5$). The duration of the tail current (in the order of seconds) would suggest that it is occurring without the presence of the orthosteric ligand (which would be washed away). As ondansetron competitively binds at the orthosteric site (Duffy *et al.*, 2012) this strongly argues that Cl-indole interacts with the orthosteric site.

These data demonstrate that antagonism of the orthosteric site prevents Cl-indole-evoked tail currents.

We have previously demonstrated that Cl-indole (in the absence of 5-HT) at concentrations up to 100 μ M does not compete with [3 H]-granisetron binding at the 5-HT_{3A} receptor (Newman *et al.*, 2013), which was confirmed in the present study (Figure 8). However, in the presence of sub-maximal concentrations of 5-HT, Cl-indole competed for [3 H]-granisetron binding sites, with the affinity of Cl-indole increasing for the [3 H]-granisetron-labelled orthosteric site of the 5-HT_{3A} receptor binding site with increasing concentrations of 5-HT (Figure 8, Table 1). In contrast, parallel experiments demonstrated that sub-maximal concentrations of 5-HT failed to modify the affinity of the partial agonist phenylbiguanide (Figure 8, Table 1).

In complementary experiments we tested the ability of Cl-indole to bind to the 5-HT binding protein. This protein, formed by mutation of the acetylcholine binding protein,

is able to bind 5-HT albeit with reduced affinity compared to the native 5-HT₃ receptor (Kesters *et al.*, 2013). At higher concentrations, CI-indole competed with [³H]-granisetron but did not show the same increase in affinity in the presence of 5-HT as for the homomeric receptor (supplementary data figure 1), which suggests the 5-HTBP does not fully replicate the interaction of CI-indole with the h5-HT₃A receptor.

CI-indole does not induce tail currents in the heteromeric 5-HT₃AB receptor

In contrast to the 5-HT₃A receptor, the heteromeric 5-HT₃AB receptor has been reported to have only one orthosteric site, formed at the interface of two A subunits (Miles *et al.*, 2013). Since the action of CI-indole requires the presence of the orthosteric agonist, we hypothesised that the agonist activity of CI-indole would not be apparent at the heteromeric receptor. Unlike the homomeric receptor, the presence of CI-indole (10 µM) did not induce a tail current after removal of 5-HT; it did however potentiate the peak current amplitude (control 786.7±108.3 pA, CI-indole 1111.9±192.5 pA, n=6, p=0.03, Figure 9B). As subtle differences exist in the pharmacology of the heteromeric receptor, we tested if a higher concentration of CI-indole (30 µM) would induce tail currents. CI-indole did not induce a tail current but did again potentiate the peak current amplitude (control 585.1±56.2 pA, CI-indole 984.4±136.5 pA, n=5, p=0.02, Figure 9A, B). Consistent with the lack of tail current in the heteromeric receptor, the presence of submaximal concentrations of 5-HT did not modulate the affinity of CI-indole binding at the 5-HT₃AB receptor (Table 2).

Discussion

Our previous studies demonstrated that CI-indole was a potent and selective positive allosteric modulator of the 5-HT₃ receptor (Newman *et al.*, 2013). CI-indole slowed the rate at which 5-HT₃A receptor-mediated currents decayed back to baseline following a brief application of 5-HT and appeared able to reactivate desensitised receptors (Newman *et al.*, 2013). In the present study we demonstrated that CI-indole alone fails to activate the 5-HT₃A receptor, although the presence of 5-HT reveals an action of CI-indole once desensitisation had commenced (Figure 1). Based on the present and our previous studies, we therefore hypothesised that CI-indole may modulate the desensitised state of the receptor. In the present studies the mechanism of action of CI-indole was explored more fully. We used prolonged applications of the endogenous orthosteric agonist, 5-HT, to induce robust desensitisation of the 5-HT₃A receptor but CI-indole did not alter either the rate or magnitude of receptor desensitisation (Figure 2Biii) or the rate at which 5-HT₃A receptors recovered following desensitisation (Figure 3). Given that some other allosteric modulators of 5-HT₃A receptors have a profound effect on desensitisation rate (Deiml *et al.*, 2004; Kooyman *et al.*, 1993; Van Hooft *et al.*, 1997), our data suggest that CI-indole functions via an alternate mechanism.

A surprising observation from the desensitisation studies was that the presence of CI-indole revealed tail currents following removal of the 5-HT. Our unpublished data show that mutation of the 5OHI binding site in the human 5-HT₃A receptor used in this study, had no effect on the ability of CI-indole to modulate the 5-HT response. A tail current has previously been described following the treatment of mouse 5-HT₃A receptors with 5OHI (Kooyman *et al.*, 1993). High concentrations of 5OHI (10 to 50 mM) evoked a tail current superficially similar to that evoked by CI-indole in our study. However unlike CI-indole, high concentrations of 5OHI inhibited the 5-HT response and the tail currents could be ascribed to relief of 5OHI-mediated block. Similarly, 10 mM trichloroethanol also induced a tail current at mouse 5-HT₃A receptors, which was ascribed to relief from a blocked state (Zhou *et al.*, 1996). Indeed, tail currents are most commonly ascribed to the relief from non-competitive open channel block. For example, penicillin is an open-channel blocker at the GABA_A receptor, where it binds within the pore and locks the receptor into an open but blocked conformation (Rossokhin *et al.*, 2014). Removal of penicillin generates

large tail-currents as receptors transition from an open-blocked to an open, ion-permeant state (Rossokhin et al., 2014). Open channel block of the 5-HT₃A receptor by 5-HT has been described by Hapfelmeier *et al* (Hapfelmeier et al., 2003) in macroscopic currents and at the single channel level by Corradi *et al* (Corradi et al., 2009). It has been proposed that 5-HT can occupy a non-agonist binding site and block conductance through the receptor in an open-channel like manner. It is therefore possible that Cl-indole may enhance 5-HT-mediated open-channel block, thereby causing a larger proportion of receptors to exist in a blocked state during continued agonist exposure. Following agonist removal, this population of receptors would transition from blocked to the open-conductive state and produce the observed tail currents. However, our data indicates that Cl-indole does not function in this manner. Previous reports of 5-HT-mediated channel block have shown that it is only evident at concentrations exceeding 5 μ M (Corradi *et al.*, 2009; Hapfelmeier *et al.*, 2003). In the present studies tail currents were associated with lower concentrations of 5-HT, the amplitude of tail currents was not altered across the range of 5-HT concentrations investigated (1-10 μ M), and tail currents were not observed in the absence of Cl-indole. Taken together, these data indicate that Cl-indole-induced tail currents do not arise from promotion of channel block.

We propose that Cl-indole potentiates 5-HT mediated responses by a novel mechanism where in the presence of the orthosteric ligand (5-HT), Cl-indole can bind to orthosteric sites leading to increased receptor activity. Several observations support this hypothesis: (1) the concentration-dependent presence of 5-HT reveals an ability of Cl-indole to compete for the [³H]granisetron-labelled orthosteric binding, (2) the orthosteric 5-HT₃ receptor antagonist, ondansetron, effectively blocks the Cl-indole-dependent tail current and (3) the IV curve of the tail current is indistinguishable from that for the 5-HT₃A receptor. Additional support is lent by the observation that in a heteromeric 5-HT₃AB receptor with potentially only one available orthosteric site, no tail current was observed. Our interpretation is therefore that Cl-indole acts as both a positive allosteric modulator and an orthosteric agonist requiring the presence of an orthosteric ligand such as 5-HT.

Ago-allosteric modulators have been defined as ligands that can function both as an agonist in their own right and as an allosteric modulator of the orthosteric ligand (Schwartz *et al.*, 2007). This class of modulators has been well described for GPCRs

(Smith *et al.*, 2011a; Smith *et al.*, 2011b; Yamazaki *et al.*, 2012) but has also been described for cys-loop LGICs. Thus phenobarbital, 4BP-TQS and thymol and carvacrol have all been described as ago-allosteric modulators for GABA_A, nicotinic $\alpha 7$ and 5-HT_{3A} receptors, respectively (Amin *et al.*, 1993; Gill *et al.*, 2011; Lansdell *et al.*, 2015). However, all these modulators act as allosteric agonists in the absence of the orthosteric ligand. Interestingly, 4BP-TQS acts via a site in the TM2 domain of the nicotinic $\alpha 7$ receptor (Gill *et al.*, 2011), while thymol and carvacrol display agonism via a site in TM1 of the 5-HT_{3A} receptor (Lansdell *et al.*, 2005).

The proposed mechanism of action of CI-Indole shows some similarity to compounds that modulate nAChRs by acting through 'non-canonical' extracellular agonist binding sites (Short *et al.*, 2015) or as 'accessory site-selective agonists' (Wang *et al.*, 2015). These compounds act at heteromeric receptors with the mechanism of action explained by compounds interacting with sites distinct from the conventional orthosteric site. Importantly, this study examined the effect of CI-Indole on homomeric 5-HT_{3A} receptors, which are expected to have five identical agonist binding sites.

Therefore CI-indole fundamentally differs from previously described modulators by its agonist action at the orthosteric site, as well as its allosteric action, both of which require the additional presence of an orthosteric ligand such as 5-HT. Since the action of CI-indole is hidden, requiring the presence of an orthosteric agonist to be revealed, we propose that it forms a new class of action that we term cryptic orthosteric modulation (COM).

The 5-HT_{3A} receptor has five orthosteric sites (Barnes *et al.*, 2009) of which two to three are believed to be occupied for maximal receptor activation (Corradi *et al.*, 2009). The question therefore arises whether CI-indole binds to unoccupied orthosteric sites or does it compete with 5-HT for occupied sites? Both in this study and previously (Newman *et al.*, 2013) we have shown that CI-indole potentiates responses induced by sub-maximal but not maximal 5-HT concentrations. Studies investigating receptor stoichiometry using nAChR suggest that agonist binding to a single binding site within the LGIC complex can activate the channel (Andersen *et al.*, 2013; Rayes *et al.*, 2009). As the number of binding sites engaged increases, the channel can become maximally activated (Corradi *et al.*, 2009). Therefore at low

agonist concentrations it would be predicted that relatively few binding sites are occupied and the current amplitude is consequently lower. CI-indole can then interact with the free orthosteric sites to potentiate the response; this action is only revealed by the presence of 5-HT. In contrast at higher 5-HT concentrations, multiple orthosteric binding sites are occupied, effectively blocking CI-indole access to the orthosteric site. As a result CI-indole cannot potentiate the response. Once the 5-HT molecules begin to dissociate, the orthosteric binding sites become sequentially available, allowing CI-indole to bind and re-activate the channel, thus generating the evident tail current. This proposes that CI-indole can only interact with unoccupied orthosteric sites. Our previous study demonstrated that when CI-indole was applied to desensitised receptors, a current was evoked (Newman *et al.*, 2013), which in the light of the present findings suggests that this current may have resulted from generation of a tail current.

The heteromeric 5-HT₃AB receptor has been reported to have the subunit order of A-A-B-A-B ((Miles *et al.*, 2013) Since the orthosteric site is formed from an A-A interface, this stoichiometry suggests the presence of just a single orthosteric site in the heteromeric 5-HT₃AB receptor. Our hypothesis is that the tail current is due to agonism of the receptor via the orthosteric site in the presence of the orthosteric ligand. This is supported by the absence of a tail current and lack of CI-indole competition in the presence of 5-HT for the heteromeric 5-HT₃AB receptor. Interestingly, the peak 5-HT₃AB heteromeric receptor current was potentiated by CI-indole. We interpret this to mean that CI-indole may have an allosteric action via the A-B and B-A interfaces. The potential for allosteric action through an A-B or B-A site is supported by Miles et al (Miles *et al.*, 2015) who showed that mcPBG can be both an agonist and allosteric modulator at the heteromeric receptor. These observations are consistent with our proposal that CI-indole forms a new class of molecule exhibiting both agonist and allosteric actions requiring the presence of the orthosteric ligand.

The novel mechanism of action of CI-indole at the 5-HT₃ receptor may offer therapeutic advantages for ligands with this mechanism at LGICs. Thus for instance a potentiation of 5-HT₃ receptor function may benefit patients with constipation-predominant irritable bowel syndrome. Furthermore, if the novel mechanism translates to other members of the LGIC receptor superfamily, then such potentiation

of GABA_A receptors for example may offer benefits for the treatment of a variety of conditions such as anxiety. Alternatively, a potentiation of nicotinic acetylcholine receptors may benefit patients with cognitive decline.

In summary, using the 5-HT_{3A} receptor as a model, this study identifies a previously unknown mode of activation of LGICs. We demonstrate that CI-indole represents a modulator requiring the presence of the orthosteric ligand for its action – a Cryptic Orthosteric Modulator. The retained physiological control via the endogenous agonist to reveal both actions may offer therapeutic advantages for drugs targeting LGICs via this mechanism.

Acknowledgements

SL was supported by a Physiological Society vacation studentship award to ADP. Unrestricted grant funding was also received from Celentyx Ltd to NMB.

Author Contribution

ADP, GG and NMB designed and analysed experiments. ADP, AR, GG, JP, NO'N, AJC, RO and SL performed experiments. ADP, GG and NMB conceived the study and wrote the manuscript. All authors discussed the results and manuscript.

Conflicts of Interest

None of the authors declare a conflict of interest.

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[5-HT] nM	IC ₅₀ (μM)	
	CI-indole	PBG
0	>100	15±2
10	77±12	14±1
30	49±12	14±1
100	27±6	15±3
200	10±1	10±2

Table 1. Ability of CI-indole and PBG to compete for [³H]-granisetron binding to the orthosteric site of the 5-HT_{3A} receptor. Data represent IC₅₀ values for the competing drugs in the absence or presence of 5-HT (mean±SEM, n=4-8).

[5-HT] (nM)	CI-indole IC ₅₀ (μM)
0	144±41
10	306±63
30	198±42
100	222±40
200	255±83
300	210±40

Table 2. Ability of CI-indole to compete for [³H]-granisetron binding to the orthosteric site of the 5-HT₃AB receptor. Data represent IC₅₀ values for the competing drugs in the absence or presence of 5-HT (mean±SEM, n=5).

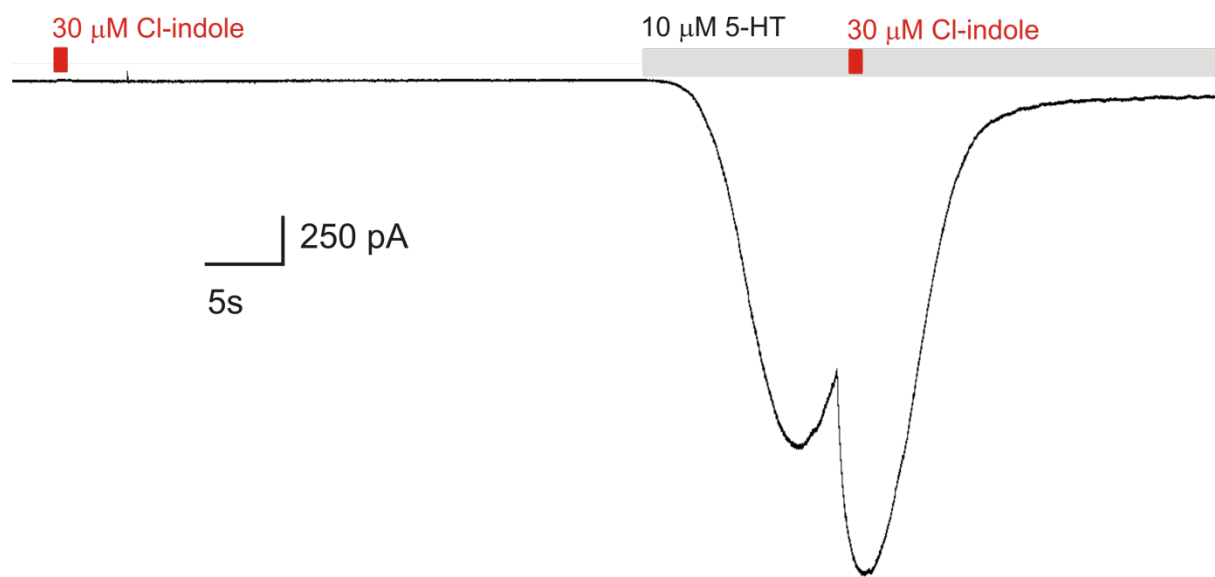


Figure 1. Cl-indole is not a 5-HT_{3A} receptor agonist

Cl-indole (30 μ M 100 ms) did not evoke a response. However in the presence of 5-HT (10 μ M, bath application), Cl-indole (30 μ M 100 ms) provoked an inward current. Representative trace, n=7.

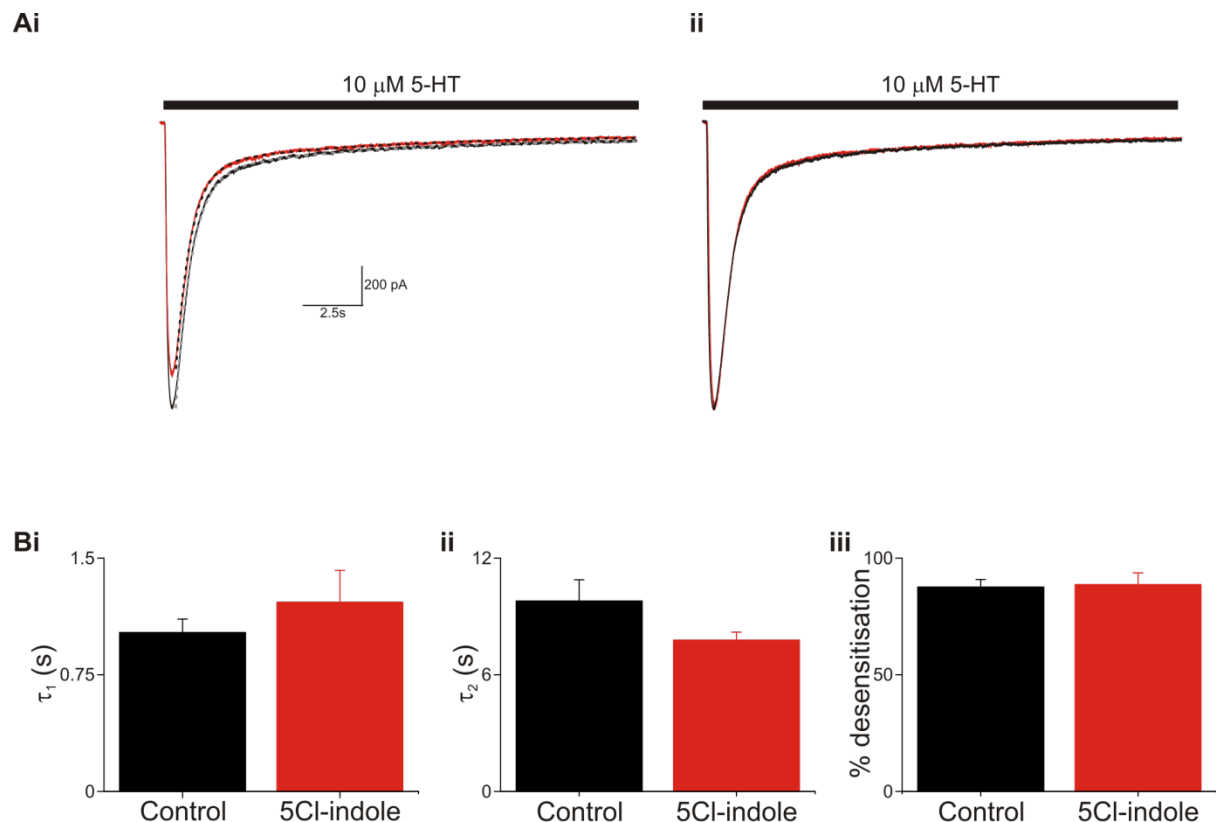


Figure 2. Cl-indole does not modulate 5-HT_{3A} receptor desensitisation (Ai); Prolonged application of 5-HT (10 μ M, 20 seconds) activated a large 5-HT_{3A} receptor current that displayed pronounced desensitisation under control conditions (black trace); single recording representative of 11 recordings. The magnitude and kinetics of desensitisation of the current evoked by 10 μ M 5-HT were unaffected by concomitant bath application of Cl-indole (10 μ M) (red trace). **(Aii)** Normalising 5-HT (10 μ M) induced current to the peak response highlighted the lack of effect of Cl-indole on receptor desensitisation. **(Bi & ii)** The time constants of the current decay in the absence (black bar) or presence (red bar) of Cl-indole (10 μ M). **(Biii)** Degree of receptor desensitisation in the absence (black bar) or presence (red bar) of Cl-indole (10 μ M). Data represent the mean \pm SEM, n=11).

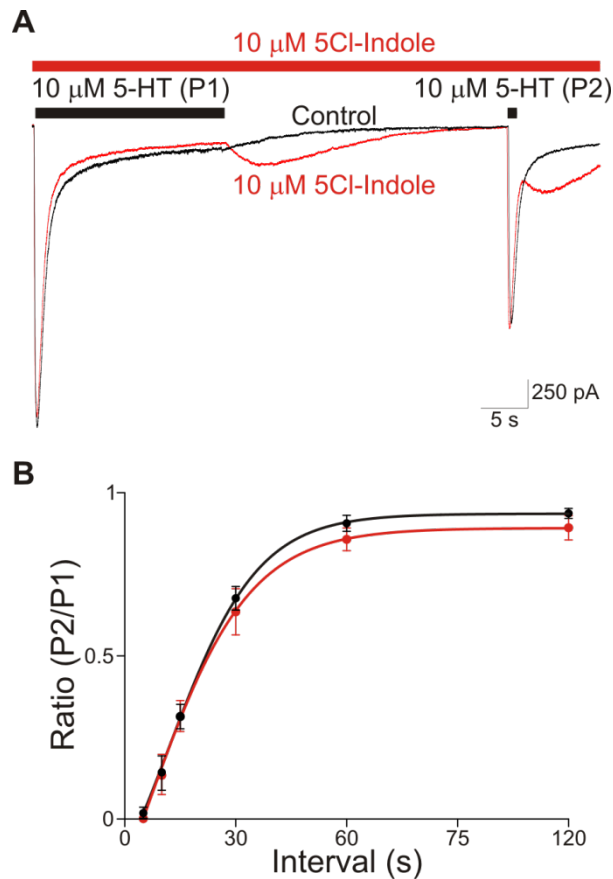


Figure 3. CI-indole does not modulate 5-HT_{3A} receptor recovery from desensitisation. **(A)** A single prolonged application (P1) of 5-HT (10 μ M, 20 s) induced similar receptor desensitisation in absence (black trace) or the presence of CI-indole (10 μ M, red trace). After removal of the 5-HT, a second test pulse (P2) of 5-HT (10 μ M, 1 s) with a delay between 5 s and 2 minutes tested recovery from desensitisation; a single representative trace from 5 recordings. Traces show an example recording with a 30 s interpulse interval. **(B)** Fractional recovery of the current amplitude elicited by the second 5-HT pulse (P2/P1) was plotted against the inter-pulse interval in the absence (black symbols) or presence of CI-indole (10 μ M, red symbols); data represent the mean \pm SEM, n=5.

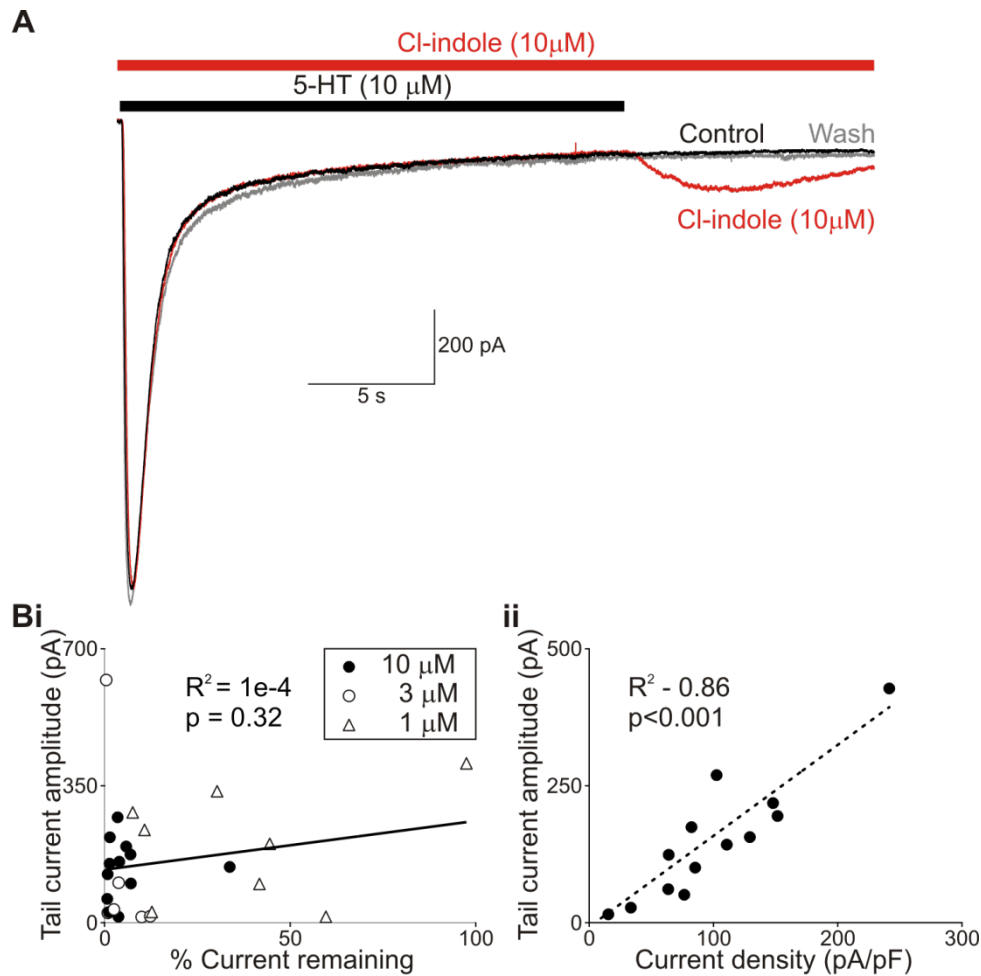


Figure 4. CI-indole evokes tail currents (A) In control conditions, the current decayed back to baseline levels after removal of 5-HT (black trace). In contrast, removal of the agonist in the presence of CI-indole (red trace) induced a tail current with slow activation and deactivation kinetics. The presence of the tail current was completely reversible upon removal of CI-indole (grey trace); trace representative of 13 recordings. **(B)** Peak tail current amplitude did not correlate with the degree of receptor desensitization **(Bi)** resulting from a 20s 5-HT application (● - 10 μ M, ○ - 3 μ M, △ - 1 μ M), but correlated with 5-HT-induced current density **(Bii)**. Data represent individual recordings.

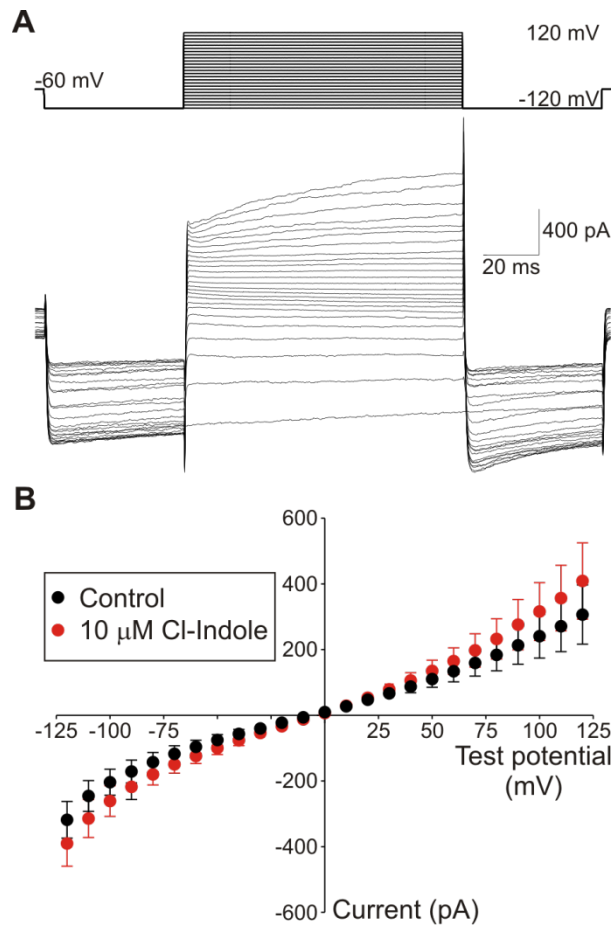


Figure 5. CI-indole does not modulate the current voltage relationship of the 5-HT₃A receptor current. (A) IV curves (-120 mV – 120 mV) were constructed at the peak of the CI-indole-induced tail current. (B) The current voltage relationship was unaltered by CI-indole (10 μ M). Data represent the mean \pm SEM, n=6.

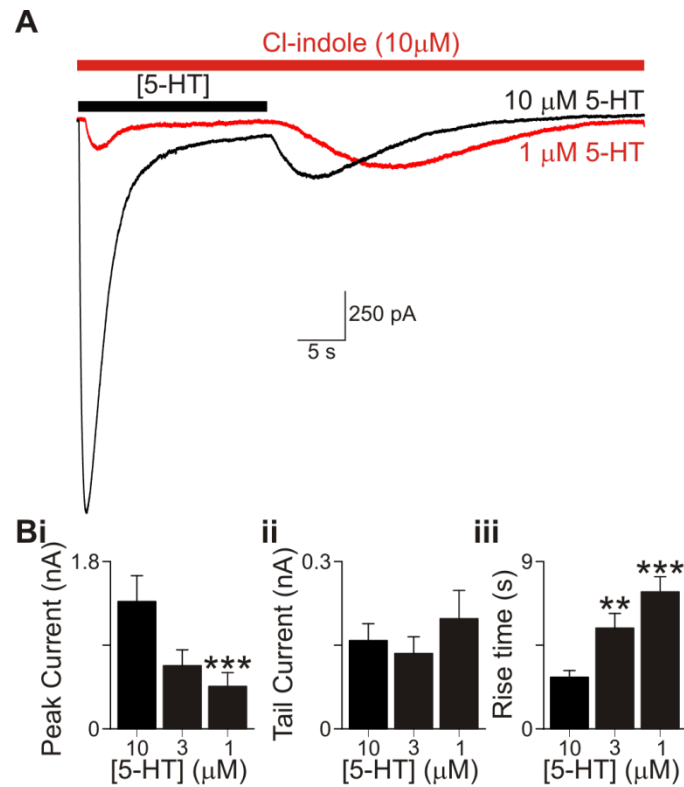


Figure 6. CI-indole induced tail current amplitude is independent of 5-HT concentration.(A and Bi) In the presence of CI-indole (10 μ M), application of 5-HT evoked a concentration dependent inward current (10 μ M – black trace, 1 μ M – red trace; trace representative of 9 recordings). (A and Bii) A similar amplitude tail current was observed at all agonist concentrations. (Biii) The rise time of the tail currents was dependent on 5-HT concentration. Bar graphs represent mean+SEM, n=9.

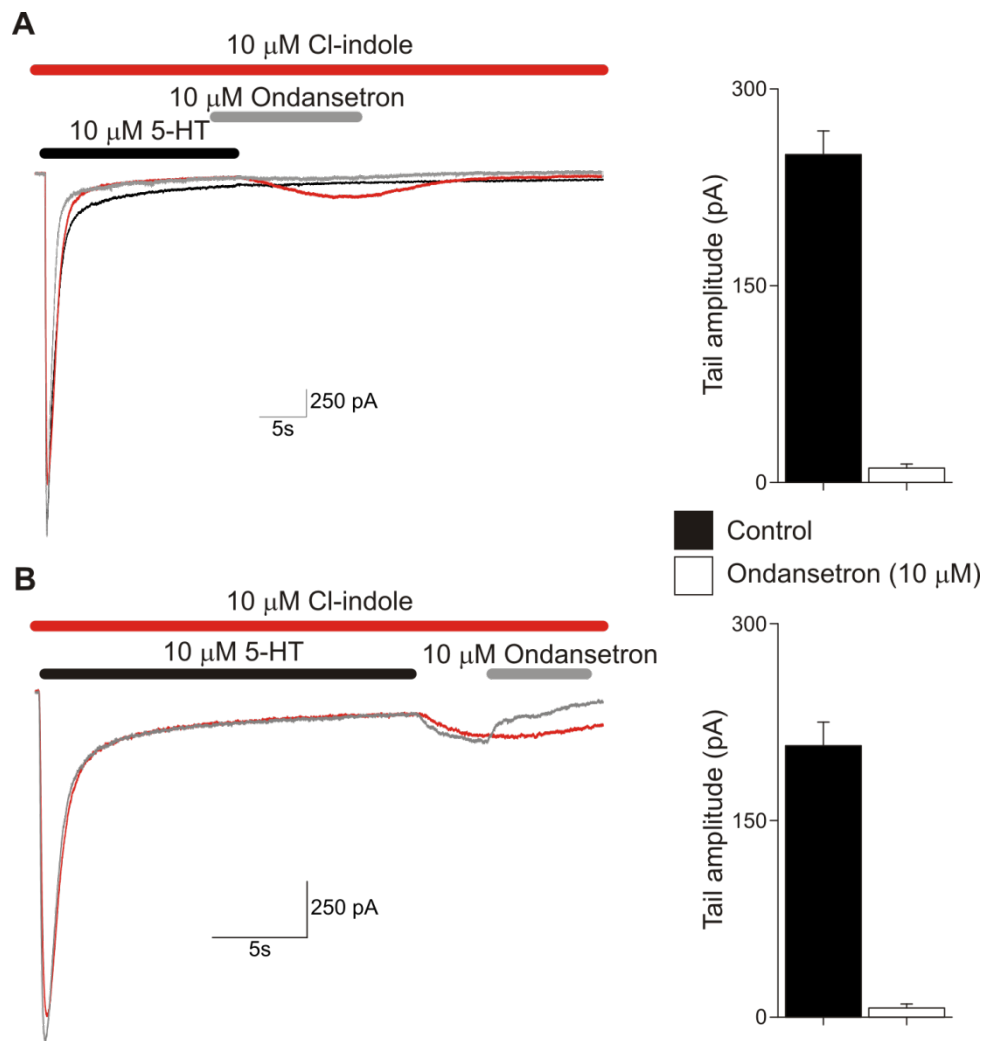


Figure 7. 5-Cl-indole mediated tail currents are blocked by ondansetron. (A) Selective orthosteric antagonist, ondansetron (10 μ M, 15 s), applied one second prior to removal of 5-HT (10 μ M, 20 s), completely blocked tail currents (grey trace), which are usually seen in the presence of 5-Cl-indole (red trace). (B) Ondansetron (10 μ M, 10 s) applied at the peak of the tail current, completely blocked the tail current (grey trace). Traces representative of 5 recordings. Bar graphs represent mean+SEM, n=5.

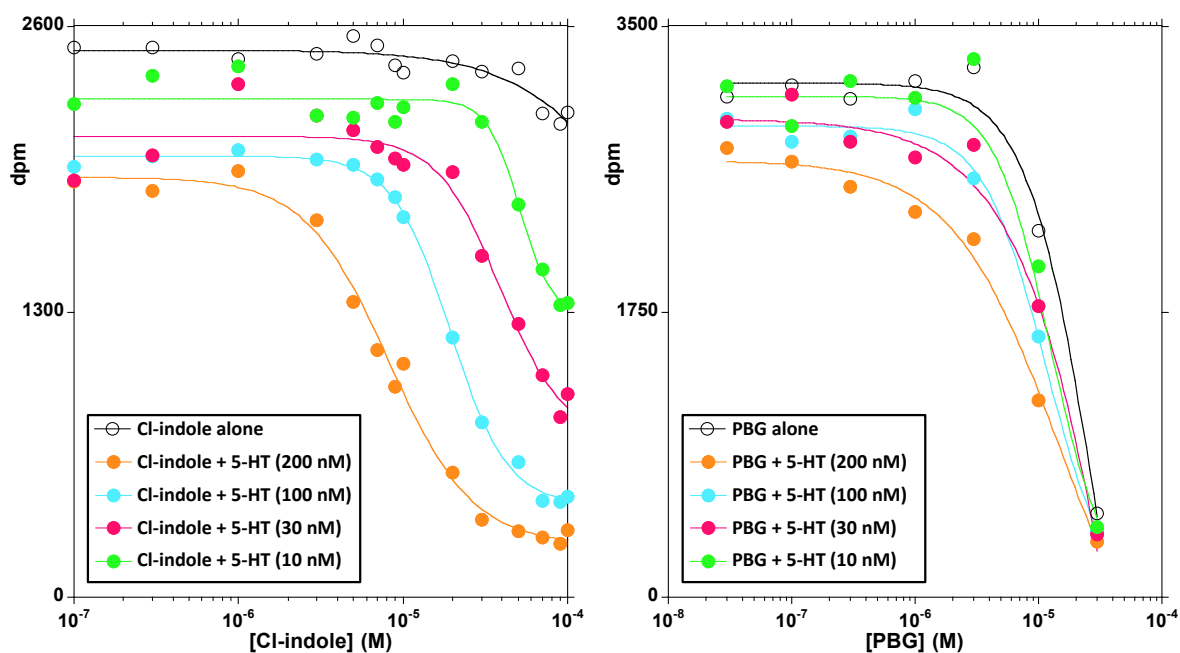


Figure 8. The presence of 5-HT allows CI-indole to compete for [3 H]-granisetron binding to the orthosteric site of the 5-HT₃A receptor. Left graph: CI-indole (up to 100 μ M) essentially does not compete for [3 H]-granisetron in the absence of 5-HT, but in the presence of 5-HT (10 – 300 nM), there is a concentration-dependent increase in the affinity of CI-indole for the [3 H]-granisetron-labelled orthosteric site. Right graph: the same concentrations of 5-HT fail to alter the affinity of the orthosteric agonist, PBG. Data from a single experiment that was representative of 4-8 independent experiments (see Table 1 for summary of arising data).

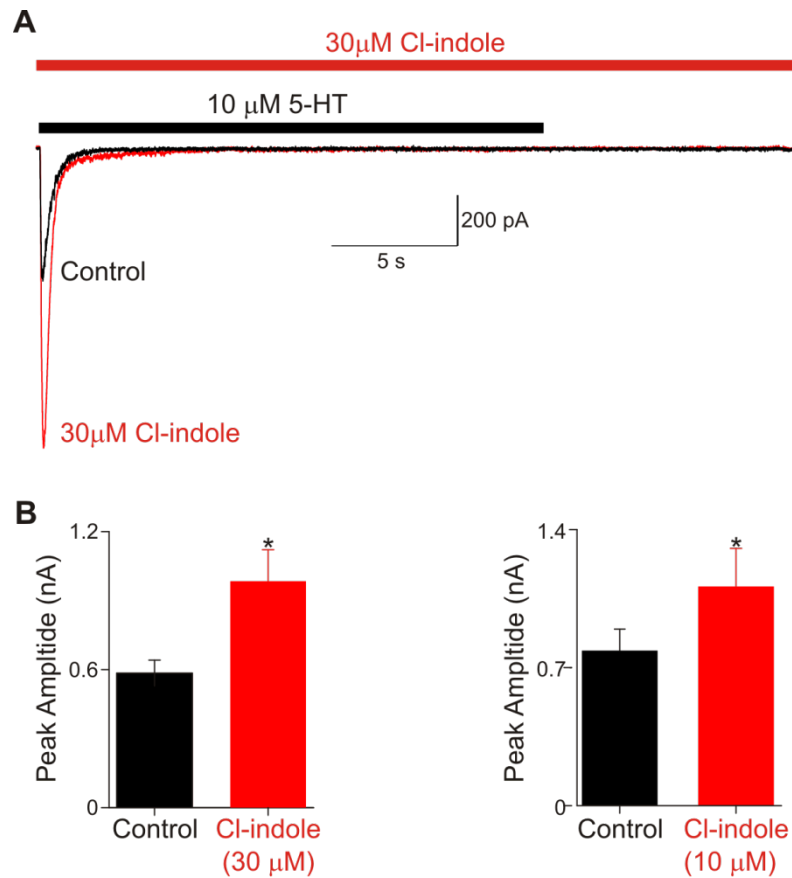


Figure 9. CI-indole does not induce tail currents in heteromeric 5-HT_{3AB} receptors. (A) Pressure ejection of 5-HT (10 μ M, 20 s, 20 psi) evoked a rapidly desensitising current in both control (black trace) and in the presence of 30 μ M CI-indole (red trace). Removal of 5-HT did not induce a tail current in either condition. (B) CI-indole (30 μ M, left panel, n=5; 10 μ M right panel, n=6) potentiated the peak 5-HT current amplitude. Bar graphs represent mean+SEM.